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[From the Physiological Laboratory of the Johns Hopkins University.]

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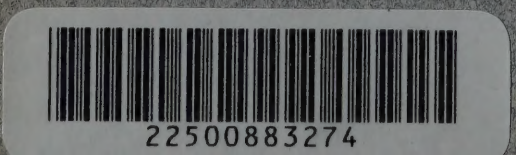

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THE RÔLE OF ANTITHROMBIN AND THROMBOPLASTIN (THROMBOPLASTIC SUBSTANCE) IN THE COAGULATION OF BLOOD.

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[*From the Physiological Laboratory of the Johns Hopkins University.*]

IN attempting to explain the coagulation of shed blood we may accept as demonstrated, in spite of the opposition of Nolf, that it is due immediately to a reaction between fibrinogen and the thrombin, whereby fibrin is formed and deposited. In the circulating blood this reaction does not occur because the thrombin though present exists in an inactive form which may be designated as prothrombin or thrombogen, and the secret of coagulation is concealed in the processes which lead to the actual production of thrombin.

Investigations by various observers have demonstrated that two factors are concerned in this formation of active thrombin. In the first place, the presence of calcium — there is practically no difference of opinion upon this point. In the absence of calcium, as in oxalated blood, the blood remains fluid indefinitely. In the second place, an unknown organic substance which may be obtained by extraction from the tissues in general, including the blood corpuscles themselves. The importance of this second factor is perhaps not so generally recognized, but to those who have given much attention to the subject it is as striking as the part taken by calcium. That this influence of tissue extracts is not due to the presence in them of thrombin or of a thrombin-like substance (coagulin) is proved sufficiently by the fact that when added to solutions of fibrinogen, with or without the presence of calcium, they do not produce a clot. The importance of the element furnished by the tissues has been emphasized greatly by a study of the blood of the vertebrates lower than the mammals. Delezenne, Nolf, and others have shown that the blood of birds, reptiles, and fishes when removed carefully from

the blood vessels, so as not to come into contact with the tissues, clots very slowly, while contact with the wounded tissues or the addition to the blood of an extract of the tissues causes it to clot in a few minutes. In fact, if in these animals the blood is removed carefully and is then centrifugalized promptly, the clear cell-free plasma clots with extreme slowness or may not clot at all if left to itself, while addition of tissue extracts causes prompt coagulation. The mammalian blood can be brought into an analogous condition by the so-called process of peptonization familiar to all physiologists. According to the success of the "peptonizing" injection the blood exhibits, when drawn at the proper period, either a delayed coagulation time of various degrees or a complete incoagulability. Such peptone bloods or peptone plasmas usually clot quickly when a tissue extract is added, behaving in this respect like the bird's plasma. The efficacy of tissue extracts in inducing or facilitating the clotting of bird's blood, peptone blood, and mammalian blood under normal conditions has found a simple and apparently satisfactory explanation in the theory proposed simultaneously by Morawitz and by Fuld and Spiro. This theory assumes that the organic substance furnished by the tissues, which has been designated by different names, is in fact an activator or kinase that converts the inactive prothrombin to thrombin. For this reaction calcium also is necessary, as is shown by the fact that bird plasma or peptone plasma which is first oxalated to remove the calcium is no longer clotted by the addition of tissue extracts. The importance of the calcium and of the tissue extract is explained on this theory by the assumption that both an inorganic (calcium) and an organic (tissue extract) activator are required to convert prothrombin to thrombin. In shed mammalian blood the organic activator (thrombokinase of Morawitz, cytozym of Fuld and Spiro) is furnished by the quickly disintegrating platelets; in the blood of the lower vertebrates similar corpuscles are lacking, and for such bloods the external tissues must supply the kinase in order to ensure rapid clotting. While this theory is simple, logically complete, and applicable in a very satisfactory way to most of the known phenomena of coagulation, it is evident to any one who gives the subject consideration that another possible explanation may be given to the effect of tissue extracts upon the clotting of bloods of all kinds. Accepting, as must be done, the facts that in the normal blood of all vertebrate

animals an antecedent or inactive form of thrombin (prothrombin) is present and also calcium salts, we may suppose that these two fail to react to form thrombin because an inhibiting substance is also present, an antithrombin, which holds the prothrombin and prevents its activation by the calcium. On this view the importance of the tissue extracts lies not in the fact that they contribute a kinase to aid in the activation of the prothrombin, but in the fact that they furnish a substance, which may be designated as a thromboplastic substance or thromboplastin, which neutralizes the antithrombin and thus permits the calcium to activate the prothrombin and start the process of clotting. All the facts that are explained on the assumption that the tissues or the blood corpuscles furnish a kinase are equally well explained on the assumption that they furnish a thromboplastic substance whose function consists in neutralizing an antithrombin, and an effort should be made to test carefully the various significant experimental facts from the standpoint of each of these possible explanations. The author has attempted to devise experiments which might serve to test these mutually exclusive views. The results are presented in the following pages. So far as the author is concerned the work has convinced him that thrombokinase, in the sense used by Morawitz, does not exist, and that the second of the two views stated is much more in accord with the facts that are known in regard to coagulation.

The Presence of Antithrombin in Peptone Plasma, in Bird Plasma, and in Normal Mammalian Plasma.

That a substance which inhibits the action of thrombin on fibrinogen is present in peptone plasma, in bird plasma, and in normal mammalian plasma is easily shown. In fact, it may be said that so far as the peptone plasma is concerned the presence of antithrombin is generally admitted, and the same is true perhaps of bird's plasma (Delezenne, Nolf, Morawitz *et al.*). Morawitz has given experiments to show also that in normal mammalian blood some antithrombin exists. For example, a serum to which some oxalated plasma has been added clots a given specimen of fibrinogen less rapidly than the same serum diluted to an equal amount with a solution of ammonium oxalate. In other words, the oxalated plasma contains something

which retards the action of thrombin on fibrinogen. The author has devised a method for obtaining pure thrombin,[1] and by making use of this preparation and of solutions of pure fibrinogen it has been easy to carry out a series of experiments which show in a conclusive way that antithrombin is a constituent of normal mammalian blood, that it is present in larger amounts in the less coagulable bird's blood and in still larger amounts in the incoagulable specimens of peptone blood obtained from dogs. In carrying out these experiments cat's blood, hen's blood, and peptonized dog's blood were used. The cat's blood and hen's blood were each received into a solution of sodium oxalate, 1 per cent, in the proportion of one part of oxalate solution to eight or nine parts of blood, and were then centrifugalized to obtain a clear and cell-free plasma. The peptonized plasma was prepared as described by Cecil in an accompanying paper by injection of the products of an acid digestion of fibrin into the artery of a dog. The blood was withdrawn in ten to fifteen minutes and was centrifugalized to obtain a clear incoagulable plasma. In order to remove from each of these plasmas its own fibrinogen (and prothrombin) it was heated for a few minutes, in a water bath, to 60° C. and then filtered from the resulting precipitate of fibrinogen. These heated plasmas had lost of course all possibility of clotting, but they still contained their antithrombin as shown by such experiments as the following.

Effect of peptone plasma heated to 60° C., and filtered. —

1. Fibrinogen, 1 c.c. Thrombin solution, five drops.	Gives a firm clot in five minutes.
2. Fibrinogen, 1 c.c. Peptone plasma, one drop. Thrombin solution, five drops.	No clot after twenty hours.

Similar preparations in which three and five drops respectively of peptone plasma were added failed to clot.

The peptone plasma was diluted with an equal volume of a solution of sodium chloride 0.9 per cent, and a preparation was made as follows:

[1] Howell: This journal, 1910, xxvi, p. 453. The principle of the method used to purify the thrombin is to remove other proteins by shaking out with chloroform. The author thought that this method was entirely new, but he has discovered since that Franz used chloroform in a similar way in the preparation of hirudin (Archiv für experimentelle Pathologie und Pharmakologie, 1903, xlix, p. 342).

Fibrinogen, 1 c.c. Peptone plasma, one drop. Thrombin solution, five drops.	The preparation clotted in one hour and thirty-five minutes.

In another series of experiments the oxalated plasma of cat's blood was used instead of solutions of fibrinogen and thrombin to test the inhibiting influence of the peptone plasma. Preparations of the following character were made:

1. Oxalated cat's plasma, ½ c.c. $CaCl_2$ (6 per cent), one drop. Peptone plasma, one drop.	Clotted firmly in five minutes.
2. Oxalated cat's plasma, ½ c.c. $CaCl_2$ (6 per cent), one drop. Peptone plasma, three drops.	Clotted firmly in seven minutes.
3. Oxalated cat's plasma, ½ c.c. $CaCl_2$ (6 per cent), one drop. Peptone plasma, five drops.	Clotted firmly in fifteen minutes.

In a second series the oxalated cat's plasma was diluted with an equal volume of glycerine and water, 1 to 3, this solution being used as a control for parallel experiments with a glycerine solution of thromboplastin to be described below.

4. Oxalated cat's plasma, ½ c.c. Glycerine and water, ½ c.c. $CaCl_2$ (6 per cent), one drop.	Clotted firmly in nine minutes.
5. Oxalated cat's plasma, ½ c.c. Glycerine and water, ½ c.c. Peptone plasma, ten drops. $CaCl_2$ (6 per cent), one drop.	Clotted firmly in eighteen minutes.
6. Oxalated cat's plasma, ½ c.c. Glycerine and water, ½ c.c. Peptone plasma, twenty drops. $CaCl_2$ (6 per cent), one drop.	Not clotted at end of one hour and imperfectly clotted the next morning (twenty hours).

These experiments with similar results reported by other observers show clearly enough that peptone plasma contains something which prevents the reaction between thrombin and fibrinogen, Since this effect is entirely removed by heating the peptone plasma to 80° to 85° C., and since it resembles otherwise the effect of known anti-

thrombin solutions (hirudin), we can explain it most acceptably by assuming that the peptone plasma contains antithrombin and indeed owes its incoagulability to the presence of this substance. The amount of it present in different specimens of peptone plasma shows naturally considerable variation.

Effect of oxalated hen's plasma heated to 60° C., and filtered. — Preparations of the following character were made:

1. Fibrinogen, 1 c.c. Thrombin solution, five drops.	Clotted firmly in eight minutes.
2. Fibrinogen, 1 c.c. Thrombin solution, five drops. Hen's plasma, five drops.	Clotted in thirty-one minutes.
3. Fibrinogen, 1 c.c. Thrombin solution, five drops. Hen's plasma, ten drops.	No clot at end of an hour, and next morning (eighteen hours) only an imperfect membranous clot.
4. Fibrinogen, 1 c.c. Thrombin solution five drops. Hen's plasma, fifteen drops.	No sign of clotting after twenty-four hours.

As proof that the inhibiting action of the hen's plasma was due to an organic substance of the nature of an antithrombin, it was then heated to 80° C., filtered, and the following preparation was made:

5. Fibrinogen, 1 c.c. Thrombin solution, five drops. Hen's plasma (80° C.), ten drops.	Clotted firmly in six minutes.

Effect of oxalated cat's plasma heated to 60° C., and filtered. — This series of preparations was made with the same solutions of fibrinogen and thrombin as those in the series just described with hen's plasma.

1. Fibrinogen, 1 c.c. Thrombin solution, five drops. Cat's plasma, five drops.	Clotted in eighteen minutes.
2. Fibrinogen, 1 c.c. Thrombin solution, five drops. Cat's plasma, ten drops.	Clotted in twenty-five minutes.
3. Fibrinogen, 1 c.c. Thrombin solution, five drops. Cat's plasma, fifteen drops.	An imperfect membranous clot formed after two hours.

The cat's plasma, as in the case of the hen's plasma, was then heated to 80° C., filtered, and tested as follows:

4. Fibrinogen, 1 c.c. Thrombin solution, five drops. Cat's plasma (80 C.), ten drops.	Clotted firmly in eight minutes.

A consideration of these and similar experiments can leave no doubt, it seems to me, that in peptone plasma, bird's plasma, and mammalian plasma there is present a substance of the nature of antithrombin. Comparative experiments with these plasmas in regard to their inhibiting effect upon the same or similar preparations of fibrinogen and thrombin make it clear also that quantitatively the amount of antithrombin is much greater in a good peptone plasma than in bird's plasma, and much greater in the latter than in mammalian (cat's) plasma. The inhibiting influence of the peptone plasma, on the other hand, is much weaker than that of a solution of hirudin made up in the strength of 0.01 gm. to 10 c.c. or 20 c.c. of water or salt solution, that is to say, a solution of 1 to 1000 or 1 to 2000. By making use of a standard solution of hirudin it would probably be quite possible to determine with some exactness the respective amounts of antithrombin present in bird's blood and mammalian blood.

The Effect of Tissue Extracts (Thromboplastin) in neutralizing the Action of Antithrombin.

As was stated in the introduction to this paper, the well-known effect of tissue extracts in causing or accelerating the coagulation of such plasmas as bird's plasma or peptone plasma may be explained either on the assumption made by Morawitz that such extracts furnish an activating substance for the prothrombin, or on the view that they contain something which neutralizes antithrombin. Experiments were made therefore to determine directly whether or not this last view is tenable.

Preparation of the thromboplastin. — The tissue extracts used in these experiments consisted of a glycerine extract of the spleen or of the thymus such as is described by Cecil in an accompanying paper. The spleen or thymus was first washed with a solution of sodium

chloride 0.9 per cent to free it from excess of blood; it was then left for twenty-four hours and ground to a fine pulp with sand. This pulp was spread in a thin layer upon glass and dried thoroughly in a current of warm air (before a register). The dried material was powdered in a mortar, was then rubbed up thoroughly with glycerine, and the glycerine preparation was centrifugalized. Experience showed that prolonged extraction with glycerine does not give so good a preparation as a rapid extraction. The centrifugalized glycerine extract seems to keep indefinitely. When used it is to be diluted with two to three times its volume of water. The dilution produces a turbidity or fine emulsion, and the solution in this condition has a strong thromboplastic action. The glycerine extracts were used as a rule in preference to aqueous or saline extracts of the fresh tissues, because they were more convenient and because in the various experiments the thromboplastic factor could be kept constant by using a single preparation throughout.

To test whether or not the thromboplastin is capable of antagonizing the action of antithrombin two general series of experiments were made. In one series hirudin was used as the antithrombin, and varying amounts of it were tested directly against mixtures of thrombin and fibrinogen or thrombin and oxalated plasma in the presence of a definite amount of thromboplastin. In the other series several different more or less indirect methods were used. The results from this latter series will be described first. In one set of experiments the antithrombin contained in the peptone plasma of dog's blood was used. The clear centrifugalized plasma was first heated for some minutes to 60° C. to get rid of its fibrinogen and prothrombin, and the filtered liquid was used against an oxalated and dialyzed cat's plasma with results of this character.

1. Oxalated plasma, 0.5 c.c. Glycerine and water (1–3), 0.5 c.c. Six per cent solution $CaCl_2$, one drop	Clotted in nine minutes.
2. Oxalated plasma, 0.5 c.c. Glycerine and water (1–3), 0.5 c.c. Heated peptone plasma (antithrombin), ten drops. Six per cent solution $CaCl_2$, one drop.	Clotted in eighteen minutes.

3. Oxalated plasma, 0.5 c.c. Glycerine and water (1–3), 0.5 c.c. Heated peptone plasma, twenty drops. Six per cent solution $CaCl_2$, one drop.	Imperfect clot after one hour.
4. Oxalated plasma, 0.5 c.c. Glycerine extract of thromboplastin (1–3), 0.5 c.c. Heated peptone plasma, ten drops. Six per cent solution $CaCl_2$, one drop.	Clotted in five minutes.
5. Oxalated plasma, 0.5 c.c. Glycerine extract of thromboplastin (1–3), 0.5 c.c. Heated peptone plasma, twenty drops. Six per cent solution $CaCl_2$, one drop.	Clotted in eight minutes.

In this experiment the retarding effect of the heated peptone (2 and 3) plasma due to its antithrombin was neutralized by the addition of the glycerine extract of thromboplastin (4 and 5). The acceleration in the time of clotting caused by the thromboplastin cannot be explained by assuming that it served to activate the prothrombin in the oxalated plasma, since the control experiment (1) with calcium chloride showed that this substance alone was sufficient for this purpose.

A second set of experiments in which hirudin was used for the antithrombin gave similar results. The hirudin was the commercial preparation and was used in solutions which contained 0.01 gm. to 10 c.c. of normal saline.

1. Oxalated plasma, 0.5 c.c. Solution NaCl, 0.9 per cent, 0.5 c.c. Six per cent solution $CaCl_2$, 1 drop.	Clotted in ten minutes.
2. Oxalated plasma, 0.5 c.c. Glycerine and water (1–3), 0.5 c.c. Hirudin, one drop. Six per cent solution $CaCl_2$, one drop.	Clot formed after one hour.
3. Oxalated plasma, 0.5 c.c. Glycerine extract of thromboplastin (1–3), 0.5 c.c. Hirudin, one drop. Six per cent solution $CaCl_2$, one drop.	Clotted in ten minutes.

Here, again, the acceleration in the clotting caused by the thromboplastin (3 compared with 2) cannot be due to its action in activating the prothrombin in the oxalated plasma, since the control (1) indicates that the calcium alone sufficed for this activation.

In a third set of experiments a preparation containing both prothrombin and antithrombin was made by heating a specimen of clear peptone plasma to 55° C. for a brief period, just sufficient to precipitate the fibrinogen; this plasma was then filtered and the following experiments were made with it and a solution of pure fibrinogen, prepared from cat's plasma. For convenience the heated peptone plasma containing prothrombin may be designated P.

1. Solution of fibrinogen, 0.5 c.c. Solution P., six drops. Six per cent solution $CaCl_2$, one drop.	Clotted in fifty-five minutes.
2. Solution of fibrinogen, 0.5 c.c. Solution P., six drops.	No clot formed.
3. Solution of fibrinogen, 0.5 c.c. Solution P., six drops. Glycerine ex't of thromboplastin, 0.5 c.c. Six per cent solution $CaCl_2$, one drop.	Clotted in nine minutes.

In these experiments the great acceleration in the time of clotting caused by the solution of thromboplastin may have been due possibly either to its hastening the activation of the prothrombin, according to the theory of Morawitz, or it may have been due to its neutralizing the influence of the anti-thrombin which was contained in solution P. That this latter explanation is the correct one is made most probable by the following additional experiments in which a solution of pure thrombin was used.

1. Solution of fibrinogen, 0.5 c.c. Glycerine and water (1–3), 0.5 c.c. Solution of thrombin, six drops.	Clotted in nine to ten minutes.
2. Solution of fibrinogen, 0.5 c.c. Glycerine extract of thromboplastin, (1–3), 0.5 c.c. Solution P., five drops. Six per cent solution $CaCl_2$, one drop.	Clotted in nine to ten minutes.

3. Solution of fibrinogen, 0.5 c.c. Glycerine and water (1–3), 0.5 c.c. Solution P., five drops. Solution of thrombin, six drops.	Clotting after one hour, clot incomplete.

In this series Experiment 3 contained the same quantity of thrombin as No. 1 and should therefore have clotted in the same time. The fact that it did not is explained by the presence of antithrombin in the solution P (prothrombin).

In Experiment 2 clotting occurred promptly, because the thromboplastin added neutralized the antithrombin in P.

If one adopts the other view, namely, that the thromboplastin solution simply accelerated the activation of the prothrombin, then the fact that in No. 3 coagulation was so much delayed is not explained, since in this experiment as much thrombin was added as in No. 1. A further demonstration that the thromboplastin acted by neutralizing the antithrombin present in P was given by an experiment in which P was heated sufficiently high to destroy its contained antithrombin (85°).

4. Solution of fibrinogen, 0.5 c.c. Glycerine and water (1–3), 0.5 c.c. Sol'n P. after heating (85°), five drops. Solution of thrombin, six drops.	Clotted in fourteen minutes.

While these experiments make it highly probable that tissue extracts influence coagulation by antagonizing or neutralizing the antithrombin constantly present in blood, it is evident that more decisive proof for this view may be obtained from experiments in which definite amounts of thrombin and antithrombin are balanced against each other in a calcium-free solution of fibrinogen or in an oxalated plasma. Under such conditions the influence of the tissue extracts should be manifest if, as is assumed, their contained thromboplastin neutralizes in some way the retarding effect exercised by antithrombin upon the union of thrombin and fibrinogen. Under such conditions it will be noted that the tissue extracts cannot act as a kinase in the sense used by Morawitz, since by his hypothesis the thrombokinase is ineffective in the absence of calcium, and in the solutions employed no calcium was added except what may have been present

in the glycerine extract of the tissue. Control experiments showed that this extract when used alone was totally unable to cause the clotting of a dialyzed oxalated plasma.

The idea of a neutralization relationship between antithrombin and tissue extracts (thromboplastin) has been tested by other observers. Fuld and Spiro [2] and Morawitz [3] state specifically that no such relationship exists, but in view of the results presented below it seems probable that in their experiments the antithrombin was used in too great a concentration. So also Schittenhelm and Bodong [4] state that tissue extracts do not prevent the inhibiting action of hirudin added to solutions of fibrinogen and serum, but if a centrifugalized hirudin plasma is used then tissue extracts do neutralize the hirudin and cause clotting. The difference in results by these two methods may have been due to the fact that in the centrifugalized hirudin plasma the hirudin was present in smaller concentration, or, as is indicated by my experiments, it may be due to the fact that the relationship between fibrinogen, thrombin, and antithrombin is not quantitatively the same in the case of solutions of fibrinogen, isolated by several precipitations, and solutions of fibrinogen as they exist normally in the blood. That is to say, the restraining effect which antithrombin exercises upon the reaction between fibrinogen and thrombin is more marked when purified solutions of fibrinogen are used than in the case of a normal plasma.

In the first experiments made by the author to test the neutralization relationship between antithrombin and thromboplastin apparently negative results were obtained owing to the fact that the solutions of antithrombin used (hirudin) were too concentrated. When this mistake was rectified by a proper dilution of the hirudin solutions, the fact that such a neutralization relationship exists was clearly demonstrated, as appears from the following series of experiments:

Experiment, April 4. — Materials used: Solution of fibrinogen, solution of hirudin (1–40,000), solution of pure thrombin, glycerine extract of thymus (thromboplastin).

[2] Fuld and Spiro: Hofmeister's Beiträge, 1904, v, p. 171.

[3] Morawitz: Archiv für klinische Medizin, lxxix, p. 432, and Ergebnisse der Physiologie, 1905, iv, p. 307.

[4] Schittenhelm and Bodong: Archiv für experimentelle Pathologie und Pharmakologie, 1905, liv, p. 217.

1. Fibrinogen, 0.5 c.c.
   Hirudin, one drop.
   Thromboplastin, 0.5 c.c.
   Thrombin, five drops.
   } Clotted in less than an hour.
2. Same mixture except hirudin, two drops, clot in three hours.
3. Same mixture except hirudin, three drops, no clot.
4. Same mixture except hirudin, four drops.
5. Same mixture except hirudin, five drops.
   } No clot.

I. Fibrinogen, 0.5 c.c.
   Hirudin, one drop.
   Glycerine and water (1–3), 0.5 c.c.
   Thrombin, five drops.
   } Clot in two hours, fifteen min.

II. Same mixture except hirudin, two drops, feeble clot in six hours, thirty minutes.

III. Same mixture except hirudin, three drops.
IV. Same mixture except hirudin, four drops.
V. Same mixture except hirudin, five drops.
   } No clot.

Comparison of Nos. 1 and 2 with I and II shows that in the presence of the thromboplastin the process of clotting was greatly accelerated. A similar result was obtained with the same mixtures in which the hirudin was twice as concentrated (1–20,000).

Experiment, May 9. — Solutions used: Oxalated plasma of cat's blood, centrifugalized and dialyzed, solution of hirudin, 1–20,000, solution of pure thrombin, saline extract of (0.7 per cent NaCl) washed terrapin's muscle (thromboplastin).

1. Plasma, 0.5 c.c.
   Hirudin, two drops.
   Thromboplastin, 0.5 c.c.
   Thrombin, five drops.
   } Clot in one-half hour.
2. Same mixture except hirudin, four drops, clot in two hours, fifteen minutes.
3. Same mixture except hirudin, six drops, clot next morning (twenty hours).
4. Same mixture except hirudin, eight drops, clot next morning (twenty hours).

I. Plasma, 0.5 c.c.
   Hirudin, two drops.
   Sodium chloride (0.7 per cent), 0.5 c.c.
   Thrombin, five drops.
   } Imperfect clot after two to three hours. Not increased after twenty-four hours.

II. Same mixture except hirudin six drops, no clot after twenty-four hours. This experiment furnishes very striking evidence of the effect

of a tissue extract in neutralizing the influence of the hirudin upon the process of clotting. The only objection that might be raised to its conclusiveness is that the saline extract of terrapin's muscle may have contained some calcium salts, and these together with the tissue extract (thrombokinase) may have activated the prothrombin in the plasma used. The very small amount of calcium in muscle makes this suggestion improbable, and it is ruled out entirely by the fact that specimens of the plasma plus the saline extract of muscle showed no signs of clotting at all.

Experiment, May 10. — The same solutions were taken as in the previous experiment except that for the thromboplastin a glycerine extract of thymus was used.

1. Plasma, 0.5 c.c.
   Thromboplastin, 0.5 c.c.
   Thrombin, five drops.
   Hirudin, four drops.
   } Clotted within thirty minutes.
2. Same mixture except hirudin, six drops, clotted in one hour, twenty-five minutes.
3. Same mixture except hirudin, eight drops, not clotted after one hour, thirty minutes, but clotted next morning (sixteen hours).

I. Plasma, 0.5 c.c.
   Glycerine and water (1–3), 0.5 c.c.
   Thrombin, five drops.
   Hirudin, four drops.
   } Not clotted after one hour, thirty minutes. Found clotted next morning (sixteen hours).

II. Same mixture except hirudin, six drops, not clotted after one hour, thirty minutes, but found clotted next morning (sixteen hours).

III. Same mixture, except hirudin, eight drops, not clotted after twenty hours.

Experiment, May 17. — Solutions used: Cat's oxalated plasma, centrifugalized and dialyzed, solution of hirudin, 1–20,000, solution of pure thrombin, glycerine extract of thymus (thromboplastin).

1. Plasma, 0.5 c.c.
   Glycerine and water (1–3), 0.5 c.c.
   Thrombin, five drops.
   Hirudin, two drops.
   } Clotted in twenty-six minutes.

I. Plasma, 0.5 c.c.
   Thromboplastin, 0.5 c.c.
   Thrombin, five drops.
   Hirudin, two drops.
   } Clotted in fifteen minutes.

2. Plasma, 0.5 c.c. Glycerine and water (1–3), 0.5 c.c. Thrombin, five drops. Hirudin, three drops.	Clotted in thirty-six minutes.
II. Plasma, 0. 5 c.c. Thromboplastin, 0.5 c.c. Thrombin, five drops. Hirudin, three drops.	Clotted in twenty-two minutes.
3. Plasma, 0.5 c.c. Glycerine and water (1–3), 0.5 c.c. Thrombin, five drops. Hirudin, four drops.	Clotted in fifty minutes.
III. Plasma, 0.5 c.c. Thromboplastin, 0.5 c.c. Thrombin, five drops. Hirudin, four drops.	Clotted in thirty minutes.
4. Plasma, 0.5 c.c. Glycerine and water (1–3), 0.5 c.c. Thrombin, five drops. Hirudin, eight drops.	Not clotted in five hours. Clotted next morning, twenty-two hours.
IV. Plasma, 0.5 c.c. Thromboplastin, 0.5 c.c. Thrombin, five drops. Hirudin, eight drops.	Clotted in three hours, thirty minutes.

This experiment exhibits very completely the influence of the thromboplastin in neutralizing the effect of antithrombin; it also shows a fact which was made evident throughout the whole series of experiments, namely, that this neutralizing action of the thromboplastin is most striking when the proportion of antithrombin used is such as to greatly slow the action of thrombin upon the fibrinogen without entirely preventing this action. On the other hand, when sufficient antithrombin was used to entirely prevent coagulation for twenty-four hours or more, it was difficult to so graduate this excess as to overcome it with a solution of thromboplastin, remembering that this latter solution was added to the extent of 0.5 c.c. It is probable that this difficulty may have been due to the fact that the thromboplastin solutions used were in reality quite dilute compared with those of the hirudin, so that in increasing the amount of the latter added to the mixtures the proportion of hirudin (antithrombin) was augmented relatively too fast.

Experiment, May 30. — Solutions used: Cat's oxalated plasma, centrifugalized and dialyzed — (forty-eight hours old) — solution of hirudin, 1–20,000 — solution of pure thrombin, extract of pigeon's testis made in solution of NaCl, 0.9 per cent with sodium oxalate added to 0.1 per cent.

1. Plasma, 0.5 c.c.
   Hirudin, one drop.
   Testis extract, 0.5 c.c.
   Thrombin, five drops.
   } Clot in ten minutes.

I. Plasma, 0.5 c.c.
   Hirudin, one drop.
   Sodium chloride and sodium oxalate, 0.5 c.c.
   Thrombin, five drops.
   } Clot in ten minutes.

2. Same mixture as (1), but hirudin, two drops, clot in thirty-five minutes.

II. Same mixture as I, but hirudin, two drops, small membranous clot in three hours. Firm clot at twenty hours.

3. Same mixture as (1), but hirudin, four drops, not clotted in twenty-four hours.

III. Same mixture as I, but hirudin, four drops, not clotted in twenty-four hours.

This experiment is of interest, since the tissue extract was made with an oxalated solution of sodium chloride, hence entirely free from available calcium. The neutralizing effect of this extract on the hirudin is, however, evident in 2 as compared with II.

A similar set of experiments was made with these same solutions except that the thromboplastin was supplied by a glycerine extract of thymus. These experiments are to be compared with I, II and III.

A-I. Plasma, 0.5 c.c.
   Hirudin, one drop.
   Thromboplastin, 0.5 c.c.
   Thrombin, five drops.
   } Clot in ten minutes.

A-II. Plasma, 0.5 c.c.
   Hirudin, two drops.
   Thromboplastin, 0.5 c.c.
   Thrombin, five drops.
   } Clot in forty minutes.

A-III. Same mixture, but hirudin, three drops. } Clot in one hour, twenty minutes.

A-IV. Same mixture, but hirudin, four drops. } Clot in three hours.

Experiment, May 31. — Solutions used: Cat's oxalated plasma (seventy-two hours old), solution of hirudin, 1–20,000, solution of pure thrombin, solution of thromboplastin, glycerine extract of thymus.

1. Plasma, 0.5 c.c.
   Hirudin, two drops.
   Glycerine and water (1–3), 0.5 c.c.
   Thrombin, five drops.
   } Clotted within fifteen minutes.

I. Plasma, 0.5 c.c.
   Hirudin, two drops.
   Thromboplastin, 0.5 c.c.
   Thrombin, five drops.
   } Clotted within fifteen minutes.

2. Same mixture as (1), but hirudin, three drops, clotted in thirty-five minutes.

II. Same mixture as I, but hirudin, three drops, clotted within fifteen minutes.

3. Same mixture as (1), but hirudin, four drops, clotted in thirty-five minutes.

III. Same mixture as I, but hirudin, four drops, clotted in forty minutes.

4. Same mixture as (1), but hirudin, five drops, not clotted after six hours, but found clotted next morning.

IV. Same mixture as I, but hirudin, five drops, clotted in one hour, fifty minutes.

5. Same mixture as (1), but hirudin, six drops, not clotted after twenty-four hours.

V. Same mixture as I, but hirudin, six drops, not clotted in five hours, but found clotted next morning.

In this series the accelerating effect of the thromboplastin is shown, except in Nos. 3 and III, in which the control clotted somewhat more promptly. Later an additional series of tests were made as follows:

1. Plasma, 0.5 c.c.
   Hirudin, two drops.
   Glycerine and water (1–3), 0.5 c.c.
   Thrombin, five drops.
   } Clotted in twenty-five minutes.

I. Plasma, 0.5 c.c.
   Hirudin, two drops.
   Thromboplastin, 0.5 c.c.
   Thrombin, five drops.
   } Clotted in fifteen minutes.

2. Same mixture as (1), but hirudin, four drops, clotted in two hours, twenty minutes.

II. Same mixture as I, but hirudin, four drops, clotted in fifty minutes.

3. Same mixture as (1), but hirudin, six drops, not clotted in twenty-four hours.

III. Same mixture as I, but hirudin, six drops, not clotted in twenty-four hours.

Taking these series of experiments as a whole, they seem to the author to show conclusively that the material in tissue extracts, thromboplastin, accelerates the act of clotting in mixtures containing antithrombin, and no explanation of this accelerating action is possible other than the view that the thromboplastin neutralizes in some way the inhibiting influence exerted by the antithrombin. Morawitz's hypothesis that the thromboplastin functions as a kinase which in combination with calcium activates some of the prothrombin present in the plasma used is not permissible for several reasons. In the first place the effect is shown also when calcium-free solutions of fibrinogen are used in conjunction with active thrombin (experiment, April 4). In the second place no calcium was present in these solutions to act with the kinase, except possibly a minute amount which may have been contained in the 0.5 c.c. of thromboplastin solution used. That the calcium, if any, contained in these solutions was not sufficient for such a purpose is indicated by the fact that the thromboplastin solutions added to the oxalated plasma in equal amounts caused no clotting whatever, and moreover in Experiment, May 30, part 1, the tissue extract was itself oxalated to remove any calcium that may have been present.

In what way the thromboplastin neutralizes the action of the antithrombin is not immediately apparent from these experiments. The simplest hypothesis is the assumption that the thromboplastin combines with the antithrombin and thus breaks up the combination between the latter and the thrombin. Observations made upon so-called peptone plasma (Fano) in which the incoagulability is due to the presence of antithrombin show that this plasma may be made to clot by simple dilution with water, by the addition of acids, by the action of carbon dioxide, etc. So also the author has shown that incoagulable terrapin's plasma, obtained by centrifugalizing the blood which has been caught without contact with the tissues, may

be made to clot on dilution with water, although dilution with normal saline (NaCl 0.9 per cent) is without effect. It would appear from these observations that in such bloods the combination between the antithrombin and the thrombin, or rather the combination between the antithrombin and the prothrombin, is one that can be dissociated with some ease, and quite possibly therefore the thromboplastin effects this dissociation, when the concentration of the antithrombin is not too great, and thus liberates the thrombin or the prothrombin as the case may be. In coagulable liquids containing fibrinogen, thrombin, antithrombin, and thromboplastin, we have the conditions for a reaction which may tend in one direction or the other according to the concentration of the reacting constituents. The thrombin may combine with the fibrinogen or with the antithrombin, the latter may combine with the thrombin or with the thromboplastin, while the fibrinogen combines only with the thrombin and the thromboplastin combines only with the antithrombin.

While the fibrinogen can combine with thrombin but not with prothrombin, there seems to be no doubt that the antithrombin combines with the prothrombin as well as the thrombin, and indeed in peptonized plasma, in bird's blood, and even in mammalian blood the antithrombin which is undoubtedly present is probably combined with the prothrombin. At various times in these experiments the author has obtained results which seemed to indicate that the combination between antithrombin and prothrombin is more easily dissociated, by thromboplastin for example, than the combination between antithrombin and thrombin.

Application of the Foregoing Results to the Normal Coagulation of Blood.

If, as the experiments given seem to prove, a relation of neutralization does exist between thromboplastin and antithrombin, and if moreover, as also seems to be clearly demonstrated, antithrombin is a normal constituent of the circulating blood in mammals as well as in the lower vertebrates, then it is evident that the alternative theory of the normal process of clotting, proposed at the beginning of this paper, is worthy of serious consideration. The hypothesis that the tissue elements or the white corpuscles of the blood furnish an enzyme

or kinase which can activate prothrombin only in the presence of, or with the assistance of, calcium does not rest upon direct evidence. To obtain direct evidence for this hypothesis, or indeed to obtain conclusive evidence against it, it will be necessary to secure pure prothrombin and ascertain whether or not it is activated by calcium salts alone. But the alternative hypothesis that the tissue extracts (including the material obtainable from disintegrating leucocytes or blood plates) promote or initiate the clotting of blood not by activating the prothrombin but by neutralizing the antithrombin has the direct experimental basis furnished in this paper.

Looking at the matter from this standpoint, the process of normal coagulation may be pictured as follows. In the circulating blood we find as constant constituents, fibrinogen, prothrombin, calcium salts, and antithrombin. The last-named substance holds the prothrombin in combination and thus prevents its conversion or activation to thrombin. When the blood is shed, the disintegration of the corpuscles (platelets) furnishes material (thromboplastin) which combines with the antithrombin and liberates the prothrombin; the latter is then activated by the calcium and acts upon the fibrinogen. According to this view the actual process of coagulation involves only three factors, fibrinogen, prothrombin, and calcium. These three factors exist normally in the circulating blood, but are prevented from reacting by the presence of antithrombin. In the bloods of the vertebrates lower than the mammals the blood when not brought into contact with the external tissues, clots very slowly, and when caught in clean vessels and promptly centrifugalized the resulting plasma, free from corpuscular elements, may be entirely incoagulable. In these animals, therefore, effective clotting, such as is necessary to protect them from fatal hemorrhage, depends upon the cooperation of the wounded tissues which furnish the thromboplastin required to neutralize the antithrombin, this latter substance being present in these bloods in greater concentration than in the mammalian blood. From this point of view the mammalian blood differs from the bloods of the other vertebrates in that it contains within itself an available source of thromboplastin, most probably in the existence of the easily disintegrating platelets which are not found in the blood of the other vertebrates.

It will be noted that according to this hypothesis the circulating

blood contains normally the three necessary factors of coagulation and its fluidity is maintained by the presence of the antithrombin. Various observers have at times been forced to the conclusion that the circulating blood contains within itself all the factors necessary for coagulation, and the present author has called attention to an observation or experiment which seems to demonstrate this fact, as follows. If the slowly coagulating blood of the terrapin is received, without coming into contact with the tissues, into vaselined tubes, is kept at a low temperature, and while cold is thoroughly centrifugalized, a clear plasma may be obtained which will remain unclotted for days or perhaps indefinitely. Nevertheless this plasma clots very promptly upon simple dilution with water. The conditions of the experiment make it probable that none of the corpuscular elements in the blood have undergone disintegration during the process of separating off the plasma, and since the plasma coagulates upon simple dilution with water it follows that it must contain all the factors of coagulation, only under normal circumstances they are prevented from reacting. A fact of this kind is difficult to explain upon the theory of Morawitz and similar theories which assume that in the circulating blood some one factor, *e. g.*, thrombokinase, is lacking. It is explicable on the hypothesis here proposed if we assume, as is justifiable from other grounds, that the combination between antithrombin and prothrombin upon which the fluidity of the blood depends, is dissociated or weakened by dilution with water. The same suggestion may be used perhaps to explain the well-known observation of Bordet and Gengou.[5] According to these observers mammalian blood received into paraffined vessels and thoroughly centrifugalized yields a cell-free plasma which remains unclotted as long as it is retained in paraffined vessels, but clots when transferred to a glass vessel or other receptacle which the plasma can wet. This observation is in line with the numerous experiments of Nolf[6] which emphasize the importance in coagulation of the presence of finely divided particles in the coagulable liquid. Such particles, to use Nolf's phrase, act as centres of coagulation and inaugurate the process of clotting. This fact is indeed beautifully illustrated, as Rettger[7] has shown, in

[5] Bordet and Gengou: Annales de l'Institut Pasteur, 1903, xvii, p. 822.

[6] Nolf: Archives internationales de physiologie, 1908, vi, p. 115.

[7] Rettger: This journal, 1909, xxiv, p. 429.

the case of the bird's blood when withdrawn from the vessels so as not to come into contact with the tissues. This blood or the plasma obtained from it may be made to clot promptly by the addition of mechanical particles such as dust, bits of feathers, etc. All of these observations upon the accelerating effect of surface contact may be explained on the hypothesis that the combination of antithrombin and prothrombin is thereby dissociated. It is to be noted, however, that this physical factor or the effect of dilution is effective only in those plasmas in which the amount of antithrombin is small. A good peptonized plasma or hirudinized plasma will not clot upon the addition of material particles, and with difficulty, if at all, upon dilution with water.

The view here proposed of the cause of the coagulation of shed blood assumes that the circulating blood is at all times ready to clot as soon as the antithrombin is neutralized, and it therefore exalts the importance of this latter substance. As Morawitz has stated, the variations in coagulation time in different individuals and under different conditions, normal and pathological, may well depend upon variations in the supply of antithrombin. Under normal conditions there must be sufficient antithrombin present to hold the prothrombin, and yet a large excess is promptly removed by excretion or otherwise, as is shown by the fact that in peptonized dogs the diminished coagulability of the blood, caused by the over-production of antithrombin, disappears after an hour or so. It would appear therefore that in this relationship between antagonistic substances, as in other similar relations in the body, automatic regulating mechanisms exist that control the balance between the prothrombin and antithrombin. The fact that excess of thrombin (see accompanying paper by Davis) or of prothrombin yielding material (leucocytes) when injected into the circulation causes an almost immediate output of antithrombin into the blood, suggests the possibility that a part at least of this automatic regulation may be due to the fact that the prothrombin or thrombin itself constitutes a hormone which excites the secretion of antithrombin.

Summary.

1. By the use of solutions of pure fibrinogen and thrombin it is shown that an antithrombin is present in normal mammalian plasma, as well as in bird's plasma and in the so-called peptone plasma.

2. Tissue extracts contain a substance (thromboplastin) which neutralizes the effect exerted by antithrombin upon the reaction between fibrinogen and thrombin.

3. On the basis of the foregoing results the following hypothesis is suggested to explain the normal coagulation of blood. Circulating blood contains normally all the necessary fibrin factors, namely, fibrinogen, prothrombin, and calcium. These substances are prevented from reacting, and the normal fluidity of the blood is maintained, by the fact that antithrombin is also present, and this substance prevents the calcium from activating the prothrombin to thrombin. In shed blood the restraining effect of the antithrombin is neutralized by the action of a substance (thromboplastin) furnished by the tissue elements. In the mammalia the thromboplastin is derived, in the first place, from the elements of the blood itself (blood platelets). In the lower vertebrates the supply of this material, in normal clotting, comes from the external tissues.

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